Determination of the Chemical Mechanism of Neurotransmitter Receptor-mediated Reactions by Rapid Chemical Kinetic Methods[a]

GEORGE P. HESS,[b] LI NIU, AND RAYMOND WIEBOLDT

Section of Biochemistry, Molecular and Cell Biology
Division of Biological Sciences
216 Biotechnology Building
Cornell University
Ithaca, New York 14853-2703

To understand the brain, we must know how nerve cells behave and how they interact.

Francis Crick
The Astonishing Hypothesis[1]

How do nerve cells receive information from within and without the organism? How can they store information for short and long periods of time? What role do chemical reactions play in the perception and storage of information? Signal initiation between cells of the nervous system is regulated to a large extent by membrane-bound proteins, the neurotransmitter receptors. The proteins in the membrane of one cell bind chemical signals, neurotransmitters, released by a neighboring cell. The reactions involve ligand-binding steps and conformational changes of the protein. Upon binding a specific neurotransmitter, the receptors form transiently open transmembrane channels. Receptors for excitatory neurotransmitters allow sodium and potassium ions to pass through the open channels. Receptors for inhibitory neurotransmitters allow the passage of chloride ions. The properties of the receptor-mediated reactions are fundamental to the ability of a neuron to function. They determine the changes in transmembrane voltage that trigger signal transmission between neurons.

Many of the receptor-mediated reactions occur on a sub-millisecond time scale. Until recently, chemical kinetic techniques suitable for use with membrane-bound receptor proteins that must be studied in intact cells and vesicles in the sub-millisecond time region were not available. When it was observed that desensitization (the transient and reversible inactivation) of a neurotransmitter receptor can occur in the millisecond time region,[2,3] such techniques were developed.[3–7] They allow one to determine (1) the ligand-binding properties of the active form both before and after the receptor has desensitized,[5] (2) the rate constants for receptor desensitization,[5] and (3) the rate constants for the formation of the open transmembrane channel.[8,9]

Here we describe three aspects of our work. First, we outline why we use chemical kinetic approaches to study receptor-mediated reactions on the sub-millisecond time scale. Then we describe a newly developed chemical kinetic method, laser-pulse photolysis of caged neurotransmitters, with a time resolution on

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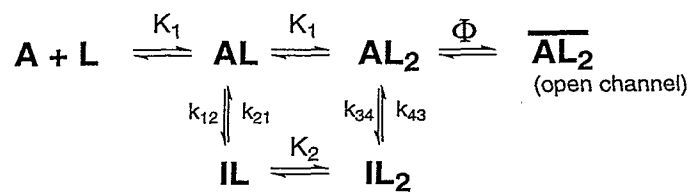
[b]Corresponding author.



SCHEME 1. Chemical mechanism for the acetylcholine receptor from *E. electricus* electroplax. A and I represent the active and inactive (desensitized) receptor forms, respectively, and K_1 and K_2 the receptor:neurotransmitter dissociation constants for the A and I forms, respectively. The subscript indicates the number of neurotransmitter molecules (L) bound. Φ is the equilibrium constant. $AL_2/\overline{AL}_2 = k_{cl}/k_{op}$ where k_{op} and k_{cl} are the rate constants for channel opening and closing, respectively. Rate constants k_{12} k_{21}, k_{34}, and k_{43} are for the interconversion between A and I receptor forms.

the millisecond scale. Finally, we describe the type of information that can be obtained.

The minimum mechanism in SCHEME 1 is based on a mechanism originally proposed for the nicotinic acetylcholine receptor in frog muscle cells by Katz and Thesleff[10] to describe the results of classical electrophysiological experiments. Once new techniques were developed the constants for the mechanism could be determined by studying the reaction before the receptor desensitized to a form with altered ligand-binding properties and biological activity. The minimum mechanism shown accounts for results obtained with (1) the muscle type nicotinic acetylcholine receptor in the electric organ of *Electrophorus electricus* (reviewed in ref. 5) and *Torpedo* sp. (reviewed in ref. 11) using quench- and stopped-flow techniques with a 5-ms time resolution; (2) the acetylcholine receptor in BC_3H1 muscle cells[4,9,12] using cell-flow[4] and laser-pulse photolysis[13] techniques with 5-ms and 100-μs time resolutions respectively; (3) the neuronal acetylcholine receptor in PC12 cells[8] using the cell-flow technique; (4) the inhibitory γ-aminobutyric type A ($GABA_A$) receptor in rat brain membrane vesicles[14,15] and in cerebral cortical cells of embryonic mice,[16] using quench-flow and cell-flow techniques with ~5- to 10-ms time resolution respectively, and (5) the inhibitory glycine receptor in embryonic mouse spinal cord cells.[17]

In the minimum mechanism in Scheme 1, A represents the receptor in its active form, and I the inactive, desensitized receptor form. The activating ligand is represented by L, and the subscript the number of ligand molecules bound to the receptor protein. $\overline{AL}_2$ represents the open-channel form of the receptor through which inorganic ions exchange across the cell membrane. K_1 and K_2 are the intrinsic dissociation constants of the ligand for the active and desensitized receptor forms respectively. We assume that each site on the active receptor form has the same affinity for ligand K_1, and that each site on the inactive form has the same affinity for ligand K_2. The channel-opening equilibrium constant Φ^{-1} was introduced[18] to account for the cooperativeness observed in the binding of neurotransmitter to the receptor. The rate constants k_{12}, k_{21}, k_{34}, k_{43} are for the interconversion between active and inactive receptor forms. The mechanism and the constants allow one to determine the concentration of the open receptor-channel, $\overline{AL}_2$ in the mechanism, as a function of neurotransmitter concentration and time.

Why is this of interest? Determination of the rate coefficient for the movement of inorganic ions across the membrane is the first step toward the calculation of the receptor-controlled change in transmembrane voltage. It is useful to look at the

underlying physical theory that relates chemical mechanisms to changes in transmembrane voltage. About 100 years ago, Max Planck[19] derived the relationship between the rate of movement of inorganic cations and anions across a porous barrier and the resulting electric field.

$$d[M^+]_1/dt = k_{obs}f_1[M^+]_2 - k_{obs}f_2[M^+]_1$$

$[M^+]$ represents the concentration of a monovalent cation, and the subscript refers to the sides of a porous barrier; and k_{obs} the voltage-independent rate coefficients for the transfer of inorganic ions across the barrier; f_1 and f_2 are the factors that account for the acceleration or retardation of the ions as they move in an electric field where $f_2/f_1 = \exp(V_mF)/(RT)$. V_m represents the transmembrane voltage, and F, R, and T the Faraday constant, the molar gas constant, and the absolute temperature, respectively. k_{obs} is given by a specific reaction rate constant, J, multiplied by the concentration of the open receptor-channel, $\overline{AL}_n$, where n represents the number of neurotransmitter molecules bound to the receptor.[20]

$$k_{obs} = J[\overline{AL}_n]_{t,L}$$

Providing we can determine J and $(\overline{AL})_n$, we can determine k_{obs}, and then calculate the transmembrane voltage.[20]

$$J = \gamma RT/F^2[M^+]NA$$

J is proportional to the conductance, γ, of the open channel. NA represents Avogadro's number and all the other terms have been defined. We can determine γ conveniently by using the single-channel technique developed by Neher and Sakmann.[21] The second task is to determine $\overline{AL}_n$ as a function of neurotransmitter concentration and time. Achieving this objective depends on knowing the chemical mechanism of the receptor-controlled reaction and the constants pertaining to it.

The interconversion between active and inactive receptor forms can occur in the 100-ms time region in the cases of the acetylcholine, $GABA_A$, and glycine receptors[3,11,14–17] and in the 10-ms time region in the case of the glutamate receptor.[22] The concentration of the open acetylcholine receptor-channel changes in the microsecond-to-millisecond time region.[23] The results in TABLE 1 illustrate why it is important to make kinetic investigations using appropriate time scales. The values were obtained with a variety of techniques in which the intermediates of the reaction are allowed to come to a quasi-equilibrium before the first measurement is made; the values differ by almost two orders of magnitude.

We decided to try a different approach. Our aim was to develop rapid chemical kinetic techniques that would allow us to investigate the sequential (pre-steady-

TABLE 1. Acetylcholine Receptor—Electrophysiological Determinations[a]

Tissue	Year	Temperature (°C)	k_{op} (s^{-1})
Frog end plate	1981	~10	2,300
	1983	~10	40,000
	1988	~10	20,000
BC_3H1 cells	1984	11	7,700
	1986	11	320
	1987	11	450

[a]Values were taken from reference 23.

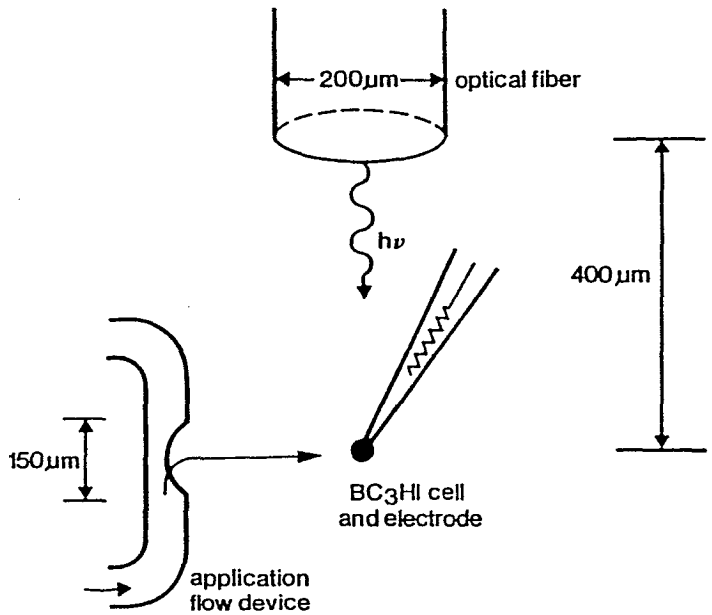


FIGURE 1A. Laser-pulse photolysis apparatus. A BC_3H1 cell, approximately 15 μm in diameter, attached to an electrode for recording whole-cell currents, was equilibrated with caged carbamoylcholine. The beam from a Candela SLL500 dye laser with a 600-ns pulse length was introduced from an optical fiber. The cell-flow method was used before and after each laser pulse to determine the concentration of liberated carbamoylcholine and to detect cell damage.

state) steps of a reaction before it reaches an equilibrium and that could be used with membrane-bound proteins in intact single cells and vesicles. What are the advantages of such an approach?

The major aim of rapid reaction techniques is to identify the sequence of reaction steps. This is a well-known and notoriously difficult problem in the kinetic analysis of quasi-equilibrium mixtures of complex reactions. However, with rapid reaction techniques it is possible to separate sequential steps of a complex reaction along the time axis. It is also possible to determine which step in a reaction occurs first, and which occurs later. Once the individual steps of a complex reaction are separated along the time axis, the kinetics for each step often follow simple rate laws. This allows one to evaluate the pertinent rate constants. Another advantage is the ability to use a wide range of reactants. A good time resolution makes it possible to use high concentrations of both receptor and neurotransmitter, thus allowing one to identify reaction intermediates that exist in such low concentrations that they may not otherwise be observed.

One of the methods we developed is a laser-pulse photolysis technique. With this it is possible to equilibrate receptors with caged neurotransmitter and then to photolyze the caged compound. This leads to the release of neurotransmitter in the μs time domain. A diagram of the apparatus is given in FIGURE 1A. A receptor-containing cell is attached to an electrode, and the solution surrounding the cell

contains a photolabile precursor of a neurotransmitter. The precursor is inactive towards the receptors so one can equilibrate it with them on the cell surface, and then release the neurotransmitter by the application of a single pulse of laser light, causing the removal of the protecting group within microseconds. FIGURE 1B is an example of the photolysis of caged carbamoylcholine which produces free carbamoylcholine plus a 2-nitrosophenyl-α-keto acid. Thus, the time resolution is improved because diffusional barriers are overcome before the activating ligand is released. The neurotransmitter binds to its specific receptors, which form open channels through which current flows. We record the resulting whole-cell current, using a technique developed by Hamill *et al.*[24]

To produce photolabile precursors of neurotransmitters, we took advantage of research on the photochemistry of *ortho*-nitrobenzyl derivatives pioneered by De Mayo in 1960,[25] Barltrop in 1966,[26,27] and Patchornik and Woodward in 1970.[28] The first applications of *ortho*-nitrobenzyl derivatives to biological problems were made by Kaplan (reviewed in ref. 29) and Trentham (reviewed in ref. 30), who synthesized photolabile precursors of biologically interesting phosphates ("caged phosphates"), for example, ATP. The compounds can be photolyzed at a rate of 10^3 s^{-1}, which is about three orders of magnitude faster than the original compounds of De Mayo[25] and Barltrop.[26,27]

We found that by attaching a carboxyl group to the benzylic carbon of the nitrobenzyl protecting group we greatly increased the photolysis rate (TABLE 2),[31] and used this approach to make photolabile precursors of carbamoylcholine. Caged carbamoylcholine is photolyzed to carbamoylcholine at a rate of 17,000 s^{-1} and with a product quantum yield of 0.8.

Unlike acetylcholine, other neurotransmitters such as γ-aminobutyric acid, glycine, and glutamate contain carboxyl groups. We could use the α-carboxy-*o*-

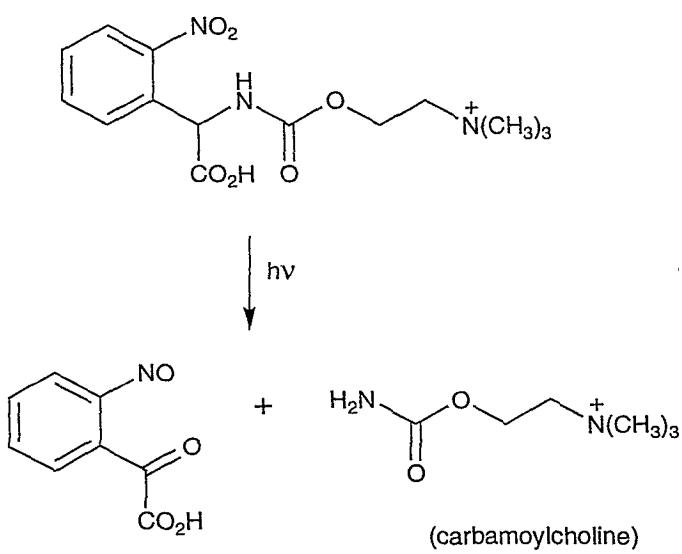
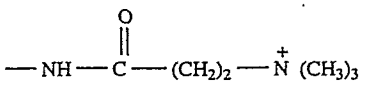
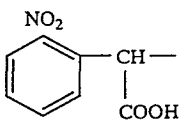
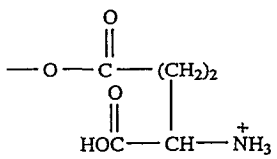
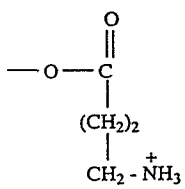
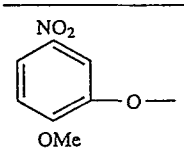
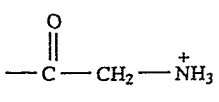


FIGURE 1B. Caged carbamoylcholine [*N*-(α-carboxy-2-nitrobenzyl)carbamoylcholine] is photolyzed to 2-nitroso-α-ketocarboxylic acid and carbamoylcholine.[31]

TABLE 2. Caged Neurotransmitters

Caging Group	Compound Caged	Photolysis Rate[a] (s^{-1})	Product Quantum Yield	Reference
	 carbamoylcholine	1.7×10^4	0.8	31
 α-carboxy-*o*-nitrobenzyl	 glutamic acid	3.3×10^4	0.14	33
	 γ-amino-butyric acid	2.3×10^4	0.15	45
 2-methoxy-5-nitrophenyl	 glycine	~70×10^4	0.2	35

[a] 22 °C, pH 7.4.

nitrobenzyl group to protect the carboxyl group of the neurotransmitters glutamate[32,33] and γ-aminobutyric acid.[34] These derivatives allow us to investigate reaction steps with rate constants as high as 20,000 s^{-1}. The derivatives are stable in aqueous solutions and are biologically inert. We have used a new photolabile group to protect the neurotransmitter glycine. The 2-methoxy 5-nitrophenyl group (TABLE 2) is photolyzed 30 times faster than the α-carboxy-*o*-nitrobenzyl group and is biologically inert.[35] It is, however, less convenient to use because it is slowly hydrolyzed in aqueous solutions at neutral pH.

What type of information can one obtain by using the laser-pulse photolysis technique? We shall discuss its use first with the nicotinic acetylcholine receptor in

BC_3H1 muscle cells and then with the glutamate receptor in hippocampal neurons. A current trace obtained in an early whole-cell experiment in which we used caged carbamoylcholine is shown in FIGURE 2A. Caged carbamoylcholine (400 μM) was equilibrated with acetylcholine receptors on the surface of a single BC_3H1 muscle cell before photolysis was induced. In this experiment, the caged derivative was photolyzed within 100 μs, and the current reached its maximum value within 2 ms. The falling phase of the current, which reflects the desensitization reaction, is shown on a different time scale.

From experiments such as the one shown in FIGURE 2A one may obtain a considerable amount of information about the mechanism. The rising phase of the current contains two types of information. At low concentrations of neurotransmitter it reflects the rate constants for the neurotransmitter-binding steps. At high concentrations of neurotransmitter it reflects the rate constants for the channel-opening process. The maximum amplitude of the current is a measure of the concentration of receptors in the open-channel form. From the effect of neurotransmitter concentration on the maximum amplitude, one can determine the equilibrium constants for neurotransmitter binding to the receptor and for the channel-opening process. Thus, the laser-pulse photolysis technique accomplishes the aim of chemical kinetic measurements. One can spread out sequential steps of a complex reaction along the time axis, so that the kinetics of individual steps can be measured separately.

The observed rate constant for the rise of the current trace in FIGURE 2A is shown as a function of carbamoylcholine concentration in FIGURE 2B. Over 90% of the rise is governed by a single exponential, suggesting that a single rate process is observed. The effect of carbamoylcholine concentration on the observed rate constant indicates that it reflects the rate constants for both channel opening and closing. The slope of the line gives a channel-opening rate constant of about 10,000 s^{-1}, and the intercept a channel-closing rate constant of 500 s^{-1}.

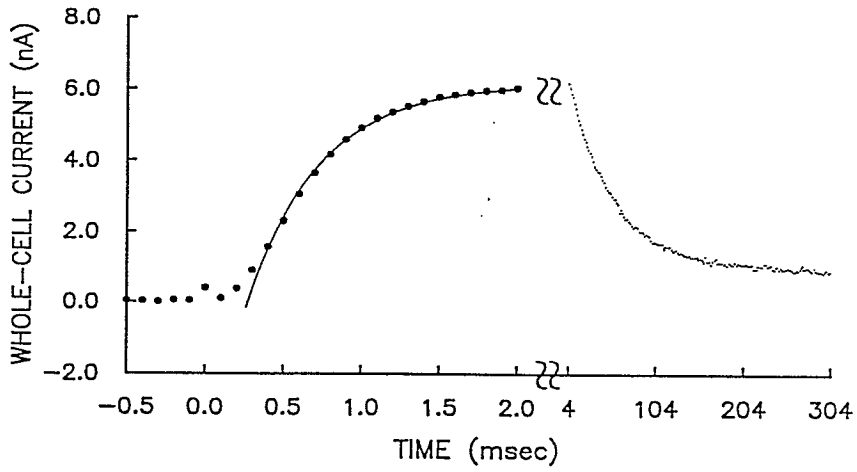


FIGURE 2A. A laser-pulse photolysis experiment with caged carbamoylcholine and a BC_3H1 cell, pH 7.4, 22–23 °C, and −60 mV. The whole-cell current was generated by photolysis of 400 μM caged carbamoylcholine. The laser excitation wavelength was 328 nm. The solid line through the points represents the rise of the current fitted to a single exponential (k_{obs} = 2140 s^{-1}).

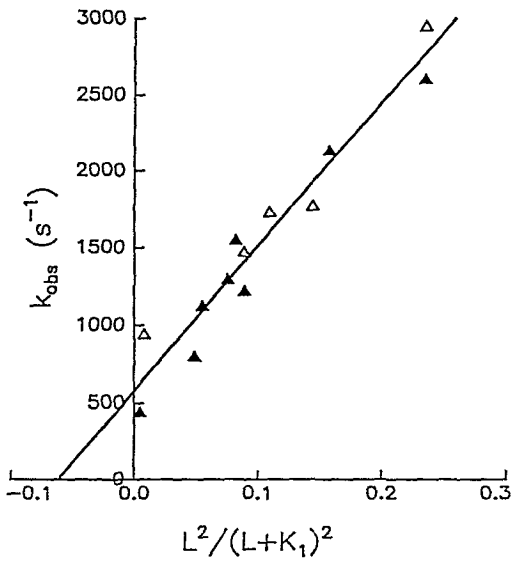


FIGURE 2B. Evaluation of the kinetic parameters for channel opening. Rate constants k_{op}, k_{cl}, and K_1 (listed in TABLE 2) were evaluated using a nonlinear least-squares fitting procedure, and the values were used to construct the solid line. The different symbols represent data from different experiments using different laser dyes with outputs at 318 or 328 nm.[12]

Can we obtain independent evidence for our measurements? Results obtained with different methods are compared in TABLE 3. All the constants obtained by laser-pulse photolysis, with the exception of k_{op}, can be evaluated by independent measurements. The rate constant for channel closing is expected to agree with the lifetime of the open channel. This lifetime was measured independently using single-channel current recordings.[12] The fraction of receptors in the open-channel form when the receptor is saturated with neurotransmitter was determined by both laser-pulse photolysis and a cell-flow technique. The value for K_1, the equilibrium constant for neurotransmitter binding to the receptor site controlling channel opening, was obtained by both photolysis and cell-flow methods. All the values obtained are in good agreement.

What other information can we obtain using the new method? An intriguing question concerned inhibition of the acetylcholine receptor by such compounds as the local anesthetic procaine,[36] the abused drug cocaine,[37] or by acetylcholine itself at high concentrations.[38] Most reports on this subject agree on a mechanism in which

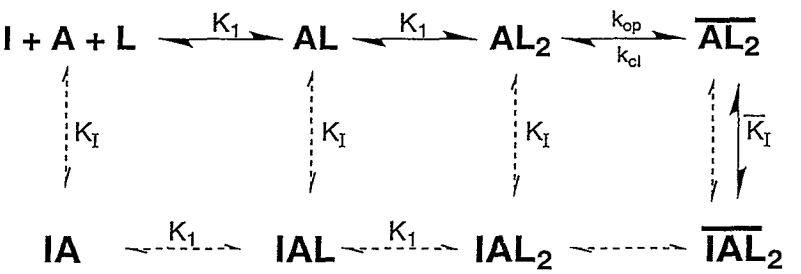
TABLE 3. Comparison of the Value of Constants Obtained with BC_3H1 Cells Using Various Methods[a]

Constant	Method	Value of Constant
k_{cl}	Laser-pulse photolysis	500 ± 100 s^{-1}
	Single-channel current	400 ± 130 s^{-1}
$k_{op}\,(k_{op} + k_{cl})^{-1}$	Laser-pulse photolysis	0.94
	Cell-flow	0.84
K_1	Laser-pulse photolysis	210 ± 90 μM
	Cell-flow	240 μM

[a]pH 7.4, 22–23 °C, −60 mV.

the receptor-channel has to open first before the inhibitor blocks the open channel.[36,38] Allosteric mechanisms in which an inhibitor binds to one form of a protein, preventing formation of an open channel, are not often invoked.

The simplest suggestion for a channel-blocking mechanism is shown in SCHEME 2. The active, non-desensitized receptor is represented by A and the neurotransmitter by L. After binding the neurotransmitter, the receptor forms an open channel, indicated by $\overline{AL_2}$. Procaine, represented by I, then binds in the open channel and blocks it. This mechanism predicts that the rate constant for channel closing will decrease as the inhibitor concentration is increased. An allosteric mechanism also accounts for all the results obtained in single-channel recordings. In the noncompetitive mechanism for allosteric inhibition, the inhibitor binds to a regulatory site on the receptor before and after the channel opens. The rate constant for channel closing is again predicted to decrease as the inhibitor concentration is increased. However, the regulatory mechanism in which the inhibitor binds to the receptor before the channel opens makes a clear-cut prediction that is not made by the channel-blocking mechanism: the channel-opening rate is expected to decrease as the inhibitor



SCHEME 2. Alternative mechanisms for inhibition of the acetylcholine receptor. L represents the channel-activating ligand. A represents the active nondesensitized receptor forms, K_1 the dissociation constant for the receptor:ligand complex, and $\overline{AL_2}$ the open-channel form of the receptor. I represents the inhibitor, and K_I and $\overline{K}_I$ the dissociation constants of the receptor: inhibitor complexes of the closed- and open-channel forms of the receptor.

concentration is increased. This is not the case with the channel-blocking mechanism. The two mechanisms can, therefore, be distinguished. However, the effect of inhibitors on the channel-opening rate constant was not measured because the time resolution of existing methods was not sufficient.

In order to distinguish between the two mechanisms we determined the effects of procaine, QX222, and cocaine on the channel-opening rate constant using the new technique. The results of two experiments done with procaine are shown in FIGURE 3. The questions we asked were: Does procaine inhibit only the channel-closing rate constant, as is predicted by the channel-blocking mechanism, or does it inhibit both the opening and closing rate constants, as is predicted by an allosteric mechanism in which the receptor has a regulatory site? The effect of procaine on k_{obs} for the current rise, measured at low concentrations of released carbamoylcholine, is illustrated in FIGURE 3; under these conditions the effect of procaine on the channel-closing rate constant, k_{cl} is determined.[9] The rate constant decreased as the procaine concentration was increased, as is predicted by both mechanisms.

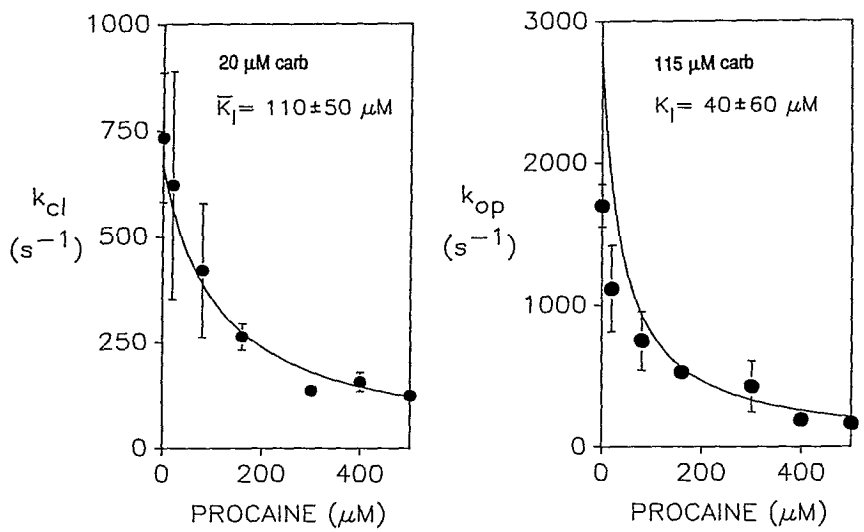


FIGURE 3. Effect of procaine on k_{cl} and k_{op} determined by the laser-pulse photolysis technique at pH 7.4, 22 °C, and −60 mV.[9]

The effect of procaine on k_{obs} for the current rise, measured at high concentrations of released carbamoylcholine, is also illustrated in FIGURE 3.[9] Under these conditions, the rate constant for channel opening[12] is much larger than that for channel closing, and k_{obs} is a measure of the channel-opening rate constant. The channel-opening rate constant decreases as the procaine concentration is increased. This is consistent with the inhibitor binding to an inhibitory site before the channel opens. Thus, the acetylcholine receptor can be inhibited before the channel opens. The new laser-pulse photolysis technique by which the constants for both channel opening and closing can be measured conveniently in the same experiment made it possible to obtain these results.

Does it make a difference which mechanism is correct? Channel opening is very fast; a receptor-channel can open in a few milliseconds, for example, the nicotinic acetylcholine receptor at a neuromuscular junction.[12,23,38,39] If the channel-blocking mechanism operates, the channel opens and a signal is initiated before the channel is blocked. Channel blocking can occur by any compound that can enter the channel but cannot pass through. The physiological meaning of the channel-blocking mechanism is, therefore, not obvious. A regulatory mechanism, in which an inhibitor binds to the receptor before the channel opens, may have physiological significance. It indicates that the receptor has a regulatory site to which specific compounds can bind and inhibit the receptor before the channel opens, thus preventing signal transmission. Acetylcholine at high concentrations inhibits the acetylcholine receptor, perhaps also by binding to a regulatory site.[40]

What other information can we obtain using a rapid chemical kinetic approach? A current trace recorded from a rat hippocampal cell and induced by photolysis of caged glutamate within ~60 μs is shown on the left of FIGURE 4A. It gives the same type of information that we obtained with caged carbamoylcholine and the acetylcholine receptor in BC_3H1 muscle cells. But we also learned something else. Caged

glutamate (500 μM) was used in this experiment. The maximum current obtained was 3000 pA, and the $t_{1/2}$ of the falling phase of the current, indicative of receptor desensitization, was about 15 ms. On the right-hand side (FIG. 4A inset) is an experiment in which 300 μM glutamate was applied to the same cell using a cell-flow device. The maximum current obtained was only 150 pA, which is 5% of the current obtained in the photolysis experiment. Furthermore, the $t_{1/2}$ of the falling phase of the current was 100 ms, which is 10 times longer than we observed in the photolysis experiment. Thus, when we used the cell-flow technique we lost all the information about the receptor form that desensitizes rapidly, because this form desensitizes during the time it takes for the receptors to equilibrate with glutamate in the flowing solution. One can improve the time resolution by using membrane patches rather than whole cells. An experiment done with a sealed membrane vesicle, with a diameter of about 7 μm, is shown in FIGURE 4B. The vesicle was suspended from a recording electrode and neurotransmitter flowed over the cell. The current induced is a measure of the concentration of open receptor-channels, and the graph shows the current produced as a function of time. The trace first rises to a maximum, within 10 ms, and then decreases due to desensitization. In this case, the rate coefficient for desensitization is about 50 s^{-1}. This means that about half the current disappears within about 15 ms; half the receptors present have desensitized within 15 ms.

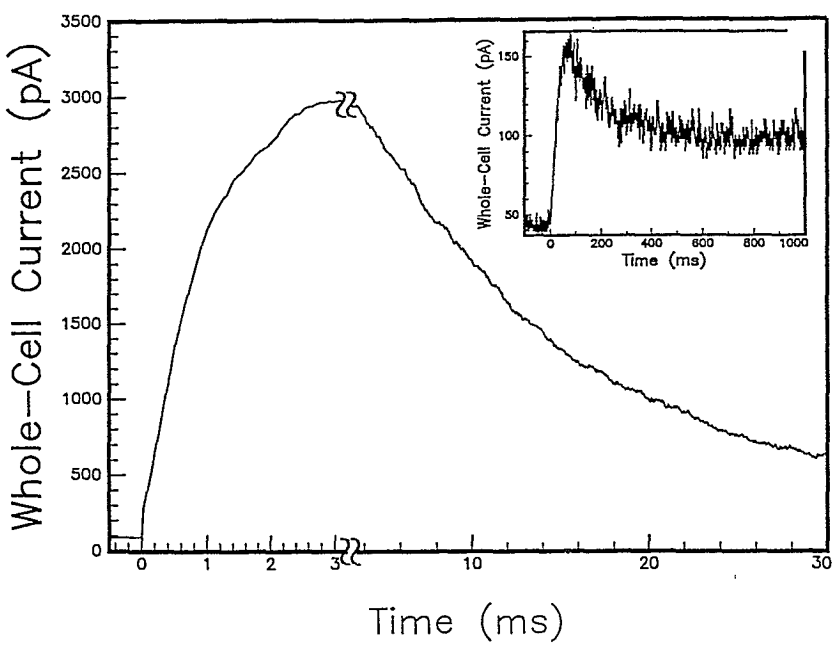


FIGURE 4A. Comparison of whole-cell current obtained with a rat hippocampal neuron by activation by laser pulse photolysis of caged glutamate and rapid flow application of a glutamate solution (pH 7.4, 22–23 °C, transmembrane voltage −60 mV). A 3-nA current is obtained from a neuron when 500 μM γ-(*o*-nitrophenyl)-α-carboxy glutamic acid is photolyzed at the cell surface by a flash of 343 nm laser light. The inset shows the response of the same neuron when it was exposed to a rapid flow of a 300 μM glutamate solution.

Even in this experiment, in which the current reaches a maximum value within 10 ms, a problem remains which has not yet been widely recognized. The receptors on the cell surface facing the flow device are in contact with the neurotransmitter and desensitize before the neurotransmitter reaches the far side of the cell. To deal with this problem, we[4] have taken into account the available hydrodynamic theory[41] to correct the observed current for the desensitization that occurs while the receptors equilibrate with the neurotransmitter. The dotted line at the top, parallel to the abscissa of the graph, gives the current after it has been corrected for desensitization. So even when a neurotransmitter equilibrates with receptors within 10 ms, as it did in this experiment, the concentration of active receptor forms can be determined only if one corrects the observed current for the desensitization that occurs during the period of equilibration. For the same reasons, the equilibrium constants that

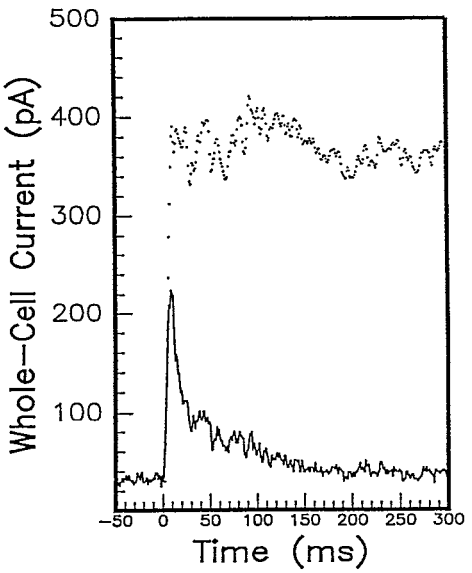
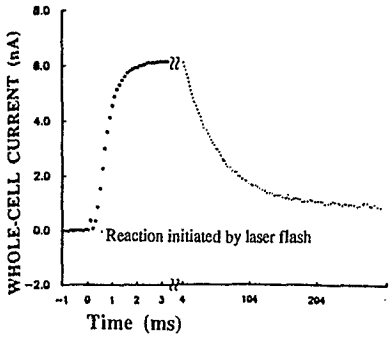


FIGURE 4B. Whole-cell recording and rapid flow of a 600-μM solution of free glutamate over a 7-μM vesicle pulled from a neuron similar to the one used for FIGURE 4A produces a reduced amplitude current response.

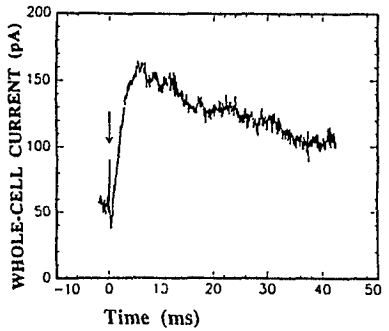
determine the concentration of open receptor-channels—which is what we want to determine—can be measured only if the observed current is corrected for desensitization. If we do not make this correction, we obtain apparent equilibrium constants, which also depend on the rate of receptor desensitization and the time resolution of the method used. The determined constants then show the same variability as they did before the use of rapid reaction techniques.

The slowly desensitizing phase of the receptors seen in the inset of FIGURE 4A is no longer seen in FIGURE 4B, because with a membrane vesicle we observe a smaller population of glutamate receptors than in a whole-cell experiments. Therefore, although we can improve the time resolution by using small membrane patches, we lose information about a minor component of the reaction. Why not use higher flow rates to improve the time resolution? First, because by using lower flow rates and

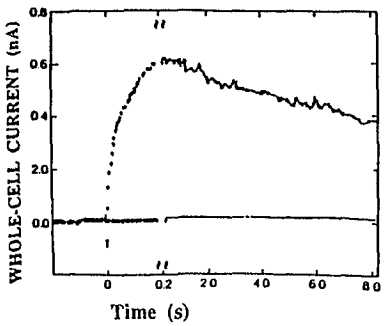
Acetylcholine receptor in BC_3H1 muscle cell
100 μM caged carbamoylchline



Glutamate receptor in mouse cortical neuron
1 mM caged glutamate



Glycine receptor in mouse spinal cord neuron
620 μM caged glycine



γ-Aminobutyric acid receptor in mouse cortical neuron
100 μM caged GABA

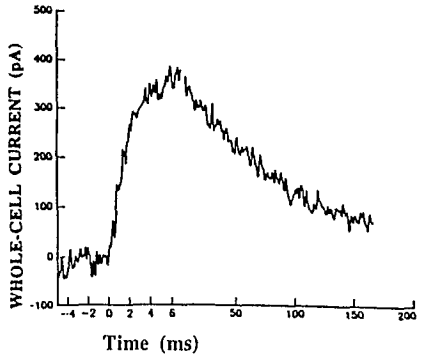


FIGURE 4C. Activation of different receptor types on mouse central nervous system neurons by photolytic release of amino acid neurotransmitters. The three caged compounds used were *N*-(α-carboxy-2-nitrophenyl)glycine, *N*-α-carboxy-2-nitrophenyl)-γ-aminobutyric acid and *N*-α-carboxy-2-nitrophenyl) glutamic acid. The experiment in the upper left corner with caged carbamoylcholine and BC_3H1 cells is the same as that in FIGURE 2A and is shown for comparison. pH 7.4, 22–23 °C, and −60 mV.

correcting the current, the seal between a cell and an electrode remains stable for much longer. This increases the number of measurements that can be made with each cell and, therefore, decreases the statistical error of the measurements. There is, however, a more serious problem. By increasing the flow rate we can shorten the apparent time it takes for a ligand to equilibrate with the receptors, but we pay a price. In the cell-flow experiments a cell is surrounded by a diffusion layer of physiological salt solution. It is important that the flowing solution containing the neurotransmitter replace the layer of buffer solution immediately surrounding the cell without mixing with it, that is to say, laminar flow is required. If the rate at which fluid flows over a cell is increased, we obtain turbulent flow. In that case neurotransmitter in the flowing solution mixes with the buffer solution surrounding the cell, and we no longer know the concentration of the neurotransmitter equilibrating with the receptors.

All the experiments we have done with the acetylcholine receptor can also be done with excitatory glutamate receptors and inhibitory GABA and glycine receptors (FIG. 4C). Very little is known about these receptor mechanisms when compared to what we know about the muscle acetylcholine receptor mechanism. All these receptors are important in controlling signal transmission. All are involved in various diseases. All are targets of clinically relevant compounds. Chemical kinetic techniques can now be used to study these processes on the molecular level.

TABLE 3 summarizes some of the results we have obtained with a cell from the central nervous system using rapid reaction techniques. We know from molecular biological experiments carried out in many laboratories that different forms of a receptor, activated by the same neurotransmitter, can coexist in one cell. Using chemical kinetic methods, we determined that different receptor forms exist in different concentrations in the same cell, that each receptor form has a different affinity for its neurotransmitter and a different rate constant for desensitization. We also have indications that the channel-opening rate constants will be different. At least eight different receptor forms may be present in a single neuron.[14–17] Some of the receptors are excitatory and some are inhibitory. All this has a significant bearing on the rate coefficients for transmembrane ion flux and, therefore, on transmembrane voltage changes and signal transmission.

Using chemical kinetic techniques, we arrived at the minimum reaction scheme shown in SCHEME 1. This scheme is based on intensive investigations of the muscle type of nicotinic acetylcholine receptor in the electric organ of two fish, *E. electricus* (reviewed in ref. 5) and *T. californica* (reviewed in ref. 11), and in BC_3H1 cells,[4,9,12] in both membrane vesicles and single cells, using a combination of rapid mixing techniques, laser-pulse photolysis, and single-channel current measurements. The same mechanism also accounts for the results of chemical kinetic investigations of a neuronal type of acetylcholine receptor in PC12 cells,[8] the inhibitory $GABA_A$ receptor in rat brain vesicles[14,15] and mouse cerebral cortical cells,[16] and the inhibitory glycine receptor in mouse spinal cord cells.[17] The overall mechanism is typical of regulatory proteins, including those that regulate metabolism and DNA biosynthesis.[42] The upper line shows the sequential neurotransmitter-binding steps leading to an open channel, which can form on the sub-millisecond time scale; an important outcome of the use of rapid reaction techniques is the determination of the constants associated with this upper line. The inactive receptor species, which can form within milliseconds, are shown on the lower line. We have determined all the rate and equilibrium constants for this mechanism, and we can predict the concentration of the open acetylcholine receptor-channel in BC_3H1 cells over a 100-fold range of neurotransmitter concentration and as a function of time.[9,12]

In this paper we illustrate the use of one of the rapid chemical kinetic techniques

we developed, and show how we evaluate some of the constants that determine the concentration of open receptor-channels over a wide range of neurotransmitter concentration and time. We give preliminary results that the diversity of receptors in central nervous system neurons can be expressed in terms of the diversity of values of the constants that determine the concentration of open receptor-channels, and indicate how this affects the transmembrane voltage changes that trigger signal initiation between neurons. We discuss a minimum reaction scheme containing the minimum number of constants, and we present one example in which chemical kinetic techniques gave additional insight into the mechanism of inhibition of the receptor-mediated reaction. We can thus learn the conditions under which a signal is transmitted by a cell in the central nervous system, and how these conditions change in response to external stimuli, diseases of the nervous system, and clinically relevant compounds. Elucidation of receptor mechanism has a bearing on many current problems in neurobiology. The results obtained with the new techniques presented previously[5–7] and here suggest that rapid chemical kinetic techniques, which are essential in elucidating mechanisms of reactions mediated by soluble proteins,[42–44] can also be applied to proteins that must be studied in a membrane-bound form in a cell or vesicle. Several methods that can be used in such studies are now available.[4,7,12] Accounting for the initiation, inhibition, and alteration of signal transmission in basic units (cells) from different areas of the nervous system in terms of well-characterized chemical reactions is, therefore, becoming an attainable goal. The new knowledge is expected to increase our understanding of the coordinated response of cells responsible for perception of and reaction to external stimuli, and integration and storage of information.

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